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TITLE: Anti-Angiogenesis by a Novel VEGF-Intrakine Strategy for  
Breast Cancer Therapy

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<p>Tumor angiogenesis plays an important role in breast cancer growth and metastasis. Vascular endothelial growth factor (VEGF) stimulates the proliferation of endothelial cells after binding to its receptor (VEGF-R), and is a key factor in tumor angiogenesis. <b>The purpose of this project</b> is to inactivate VEGF-R in vascular endothelial cells by using an intrakine/intrabody strategy to block the cell surface receptor expression intracellularly and thereby prevent endothelial cell proliferation. <b>The scope of this research</b> involves generating various expression vectors for the VEGF-intrakine/intrabody, determining their effects on the VEGF-R expression, developing an adeno-associated virus system for efficient transduction and evaluating the anti-angiogenesis activity in a mouse model. So far, immunoprecipitation, immunofluorescent and flow cytometry assays have been used to demonstrate that VEGF receptor-2 KDR is highly expressed on the surface of HUVEC. They also demonstrated the background KDR expression, which can be used as an important reference for future study of KDR expression after introducing VEGF-intrakine/intrabody into the HUVEC cell line. VEGF intrakine/intrabody expression vectors have been generated by using RT-PCR and other gene construction methods. Now the intrakine/intrabody gene can be introduced into the cells and their anti-angiogenesis function will be studied in vitro and in vivo.</p>				
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<u>Yang Wheeler</u>	06-29-2000
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## (5). Introduction:

Tumor angiogenesis plays an important role in breast cancer growth and metastasis. Vascular endothelial growth factor (VEGF) stimulates the proliferation of endothelial cells after binding to its receptor (VEGF-R) on cell surfaces, and is a key factor in tumor angiogenesis. **The subject and scope of this research** involves generating various expression vectors for the VEGF-intrakine/intrabody and determining the effects of VEGF-intrakine/intrabody on the VEGF-R expression on endothelial cell surfaces, developing an adeno-associated virus system for efficient transduction of the intrakine/intrabody gene into endothelial cells and evaluating the anti-angiogenesis and anti-tumor activity of this intrakine/intrabody approach in a mouse model. **The purpose of this research** is to inactivate VEGF-R in vascular endothelial cells by using this intrakine/intrabody strategy and thereby prevent endothelial cell proliferation, which may lay the groundwork for the development of a novel approach to breast cancer therapy.

## (6). Body:

In order to demonstrate that KDR receptor is highly expressed on the surface of HUVEC, we have used immunoprecipitation, immunofluorescent and flow cytometry assays.

Figure 1 is the result of immunoprecipitation assay. In this assay, anti-KDR antibody (Santa Cruze, sc-504), has been used to immunoprecipitate and detect KDR expression in HUVEC (ATCC) cell lysate. Two bands, 200kDa and 230kDa appeared in both VEGF stimulated and non-stimulated HUVEC cell lysate (Fig. 1). Only the 230kDa band is the mature form (1).

Figure 2 is the result of the flow cytometry assay. HUVEC and EAhy.926 (an endothelial hybridoma cell line, a gift from Dr. Cora-Jean S. Edgell in UNC Chapel Hill) were the two cell lines used in this assay. In this study, the experimental cell line has been treated with mouse anti-KDR monoclonal antibody (Sigma V9134) as the 1<sup>st</sup> antibody, and the PE-conjugated goat anti-mouse IgG as the 2<sup>nd</sup> antibody. The control cell line was treated only with the PE-conjugated goat anti-mouse IgG. Figure 2a is the result of experiments done on the HUVEC cell line. It demonstrated that there was a peak shift in the experimental group compared with the control group. The mean fluorescence has moved from 27.74 to 212.55, and 98.18% of the HUVECs had high fluorescence in experimental group, compared with only 1.37% of cells in control group. This indicates that KDR is highly expressed on the surface of the HUVEC cell line. Figure 2b is the result of experiments done on the EAhy.926 cell line. There was not a big peak shift in this cell line. The mean fluorescence has only shifted from 6.38 to 10.46, which demonstrates that there is only a small amount of KDR expression on the surface of the EAhy.926 cell line.

Figure 3 is the result of an immunofluorescent assay. The purpose of this experiment is to study KDR expression on HUVEC, HMVC cells lines and to determine whether

hypoxia can increase KDR expression. Some previous studies demonstrated that hypoxia decreased KDR expression (2), while others showed that KDR expression was increased by hypoxia (3). In our experiment, both HUVEC and HMVC cell lines had been incubated in a hypoxic incubator for 24 hours before they were incubated with anti-KDR antibody (Sigma) as the 1<sup>st</sup> antibody and Rhotamine-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) as the 2<sup>nd</sup> antibody. It has been demonstrated that there was fluorescence on both cell lines, but there was more fluorescence on normoxia treated than hypoxia treated cell lines. This indicated that hypoxia did not increase KDR expression.

In general, the above three experiments demonstrated that HUVEC can be used in this project as an ideal cell line and they also demonstrated the background KDR expression, which can be used as an important reference for future study of KDR expression after VEGF-intrakine/intrabody is introduced into the HUVEC cell line.

In order to obtain the VEGF-intrakine gene, RT-PCR has been used to obtain the VEGF<sub>121</sub> gene from HL-60 cell line (ATCC). VEGF<sub>121</sub> forward primer was: 5'- TTT, GAA, TTC, TCG, GGC, CTC, CGA, AAC, CAT, GA -3' and reverse primer was 5'- TT, TCT, AGA, GGA, TCC, TCA, CCG, CCT, CGG, CTT, GTC, ACA, TC -3'. PCR amplification was done under the following conditions: 94°C for 3 minutes for initial melting; 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds for a total of 30 cycles, and 72°C for 7 minutes for final extension. Figure 4 demonstrated the result of RT-PCR, a band around 500bp appeared on the 1% agarose gel, which is the VEGF<sub>121</sub> gene. This VEGF<sub>121</sub> was further purified from the gel and modified by adding influenza hemagglutinin (HA) marker, and/or endoplasmic reticulum (ER)-trapping signal "KDEL". Then the modified VEGF<sub>121</sub> genes were inserted into the pIRES2-EGFP plasmid. The newly constructed plasmids VEGF<sub>121</sub>-pIRES, VEGF<sub>121</sub>-HA-pIRES, VEGF<sub>121</sub>-KDEL-pIRES and VEGF<sub>121</sub>-HA-KDEL-pIRES were digested with EcoRI and BamHI (Fig. 5) and sequenced in the DNA sequencing core lab at Wake Forest University School of Medicine.

An alternative to VEGF-intrakine strategy is the VEGF-intrabody strategy. An anti-KDR single chain antibody scFv-p3S5 was obtained from Imclone company (NY). This scFv has been demonstrated to have a high affinity for the KDR receptor (4). PCR has been used to obtain the scFv-p3S5 gene from the original plasmid. Further PCR has been used to add signal sequence, influenza hemagglutinin (HA) marker and/or KDEL sequence into the scFv-p3S5 sequence. These modified scFv genes were then inserted into pIRES-EGFP vector. The newly constructed plasmids p3S5-pIRES, p3S5-HA-pIRES, p3S5-KDEL-pIRES and p3S5-HA-KDEL-pIRES were sequenced in the DNA sequencing core lab at Wake Forest University School of Medicine.

### **(7) . Key Research Accomplishments:**

- Immunoprecipitation, immunofluorescent, and flow cytometry assays have been used to determine KDR expression on the surfaces of HUVEC cell line. The results from these experiments demonstrated that the HUVEC cell line can be used for future studies and they also provided important background reference for future research.
- VEGF-intrakine and VEGF-intrabody plasmids have been generated. These plasmids will be introduced inside the cells and their function will be studied.

### **(8). Reportable Outcomes:**

Presentations:

- Annual Wake Forest University Cancer Biology Department Retreat: 08/1998
- Cancer Biology Department Journal Club: 03/2000

### **(9). Conclusions:**

Immunoprecipitation, flow cytometry and immunofluorescent assays have demonstrated that the HUVEC cell line is an ideal cell line for future study in this anti-angiogenesis project. They also provide important background reference for further study of introducing intrakine/intrabody gene into the cells. The generated intrabody/intrakine gene can now be introduced into the cells and their anti-angiogenesis function will be studied in vitro and in vivo.

### **(10). References:**

- (1). Takahashi T, and Shibuya M. The 230kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC- $\gamma$  pathway and partially induces mitotic signals in NIH3T3 fibroblasts. *Oncogene* 1997; 14 (17): 2079-1089.
- (2). Takagi H, King GL, Ferrara N, and Aiello LP. Hypoxia regulated vascular endothelial growth factor receptor KDR/Flk gene expression through adenosine A<sub>2</sub> receptor in retinal capillary endothelial cells. *Invest. Opth. & Visual Sci.* 1996; 37:1311-1321.
- (3). Rossler J, Breit S, Havers W, and Schweigerer L. Vascular endothelial growth factor expression in human neuroblastoma: up-regulation by hypoxia. *Intl. J. Cancer* 1999; 81: 113-117.

(4). Lu D, Kotanides H, Jimenez X, Zhou Q, Persaud K, Bohlen P, Witte L, and Zhu Z. Acquired antagonistic activity of a bispecific diabody directed against two different epitopes on vascular endothelial growth factor receptor 2. *J. Immunol Methods* 1999; 230: 159-171.

**(11). Appendices:**

None.

**(12). Final Reports:**

No abstract or paper has yet been published on this project.

Principle investigator (Yurong Y. Wheeler) has received student payment from this award of \$1,250.34/month for the last one year.



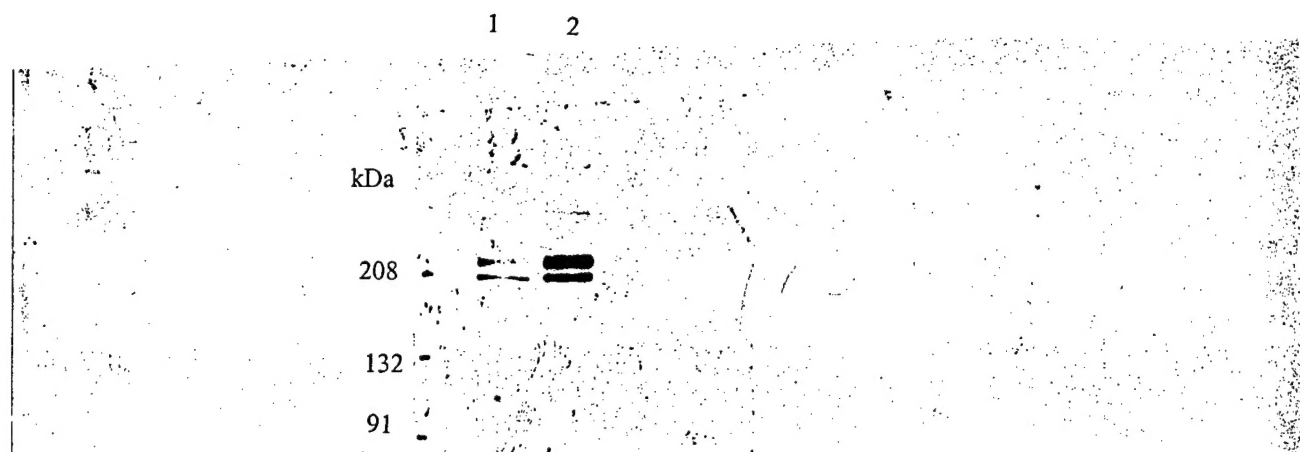


Fig. 1 Immunoprecipitation assay to detect KDR expression in HUVEC cell lysate. Two bands, 200kDa and 230kDa appeared in both VEGF stimulated (lane 1) and VEGF non-stimulated HUVEC cell lysate (lane 2). Only the 230kDa is the mature form.

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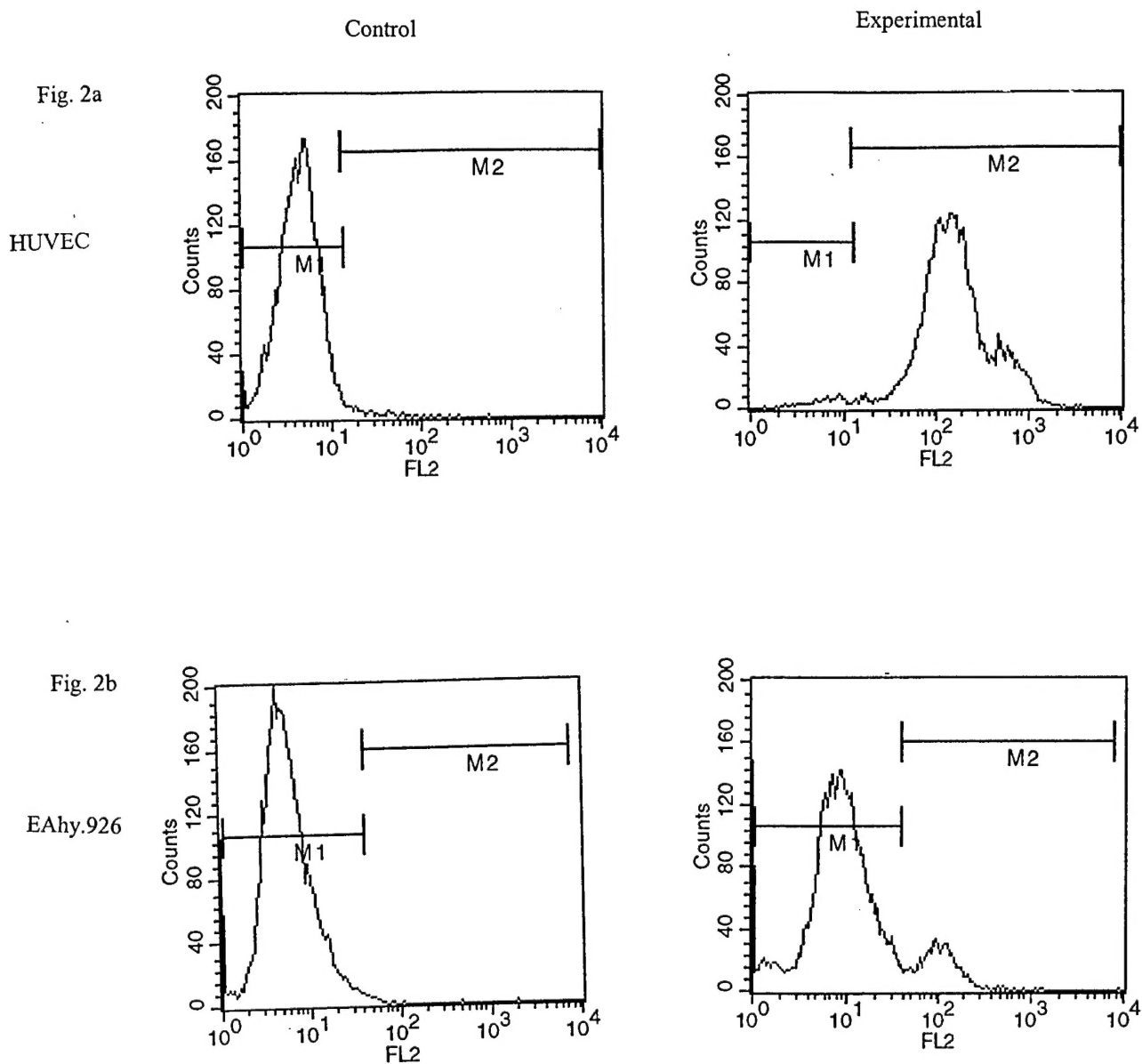


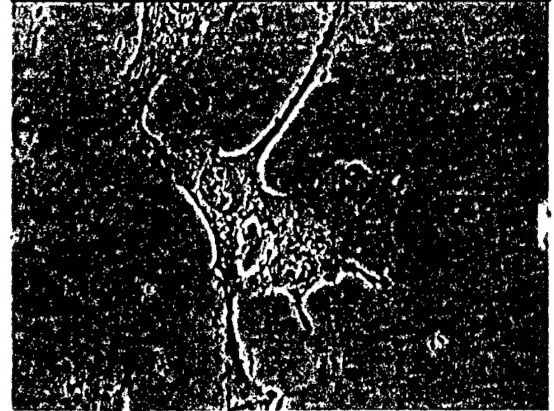
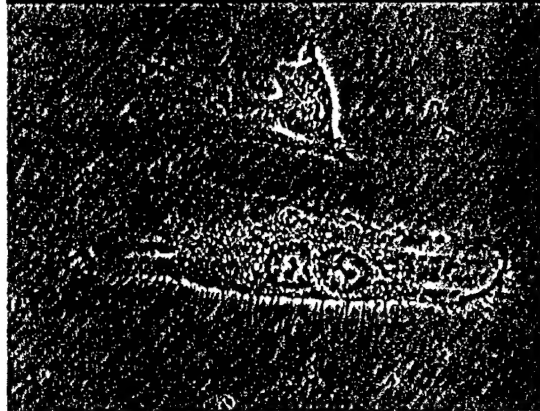
Fig. 2 Flow cytometry assay to detect KDR expression on HUVEC and EAhy.926 cell surfaces. Fig. 2a demonstrated that in HUVEC cell line, there was a peak shift in the experimental group compared with the control group. The mean fluorescence has moved from 27.74 to 212.55, and 98.18% of the HUVECs had high fluorescence in experimental group, compared with only 1.37% of the cells in control group. Fig. 2b demonstrated that in EAhy.926 cell line, there was not a big peak shift in the experimental group compared with the control group.

immunofluorescent



HMVC

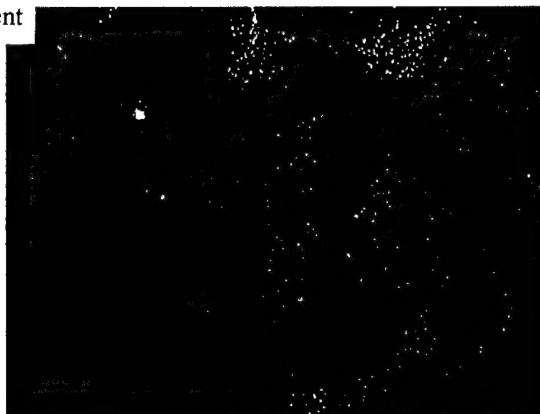
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Normoxia

Hypoxia

munofluorescent



HUVEC

Phase contract

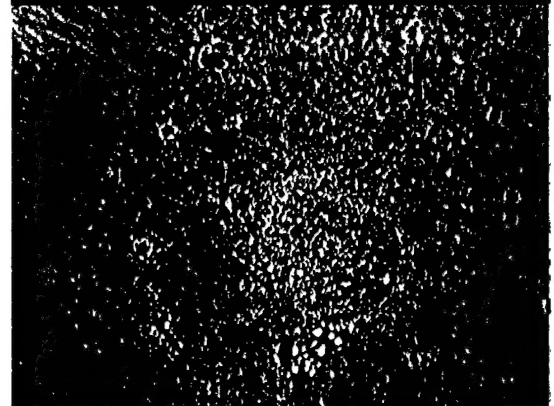
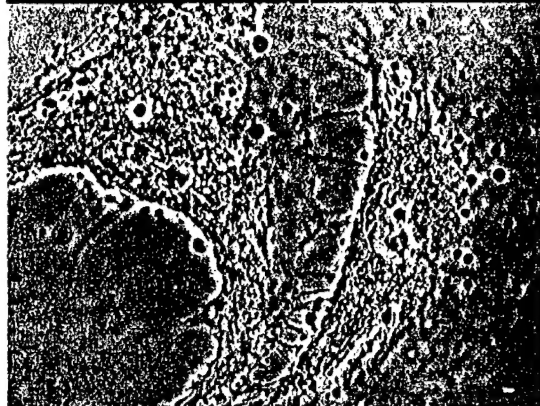


Fig. 3 Immunofluorescent assay to determine KDR expression on hypoxia and normoxia treated HUVEC and HMVC cell lines.

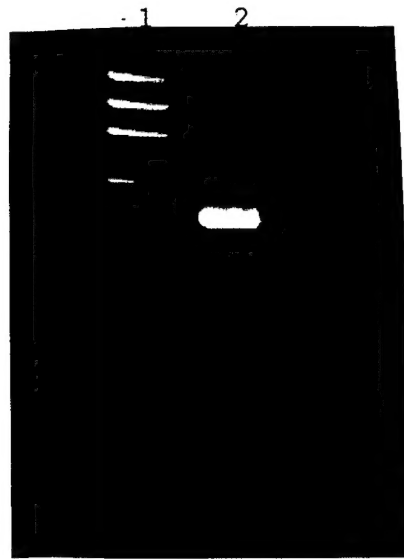


Fig.4 RT-PCR to obtain VEGF121 from HL-60 cell line. Lane 1 is the 174 Phi DNA marker. In lane 2, a band around 500bp appeared on the 1% agarose gel, which is the VEGF121 gene.

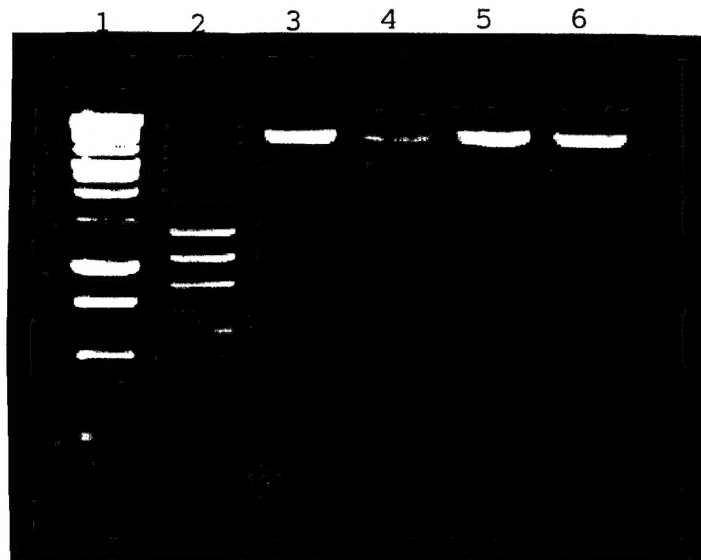


Fig.5 The newly constructed VEGF121-pIRES (lane3), VEGF121-HA-pIRES (lane4), VEGF121-KDEL-pIRES (lane5) VEGF121-HA-KDEL-pIRES (lane6) were digested with EcoRI and BamHI. Lane 1, 1kb DNA marker; lane 2, 174 phi DNA marker.